The Asian and Australasian Journal of Plant Science and Biotechnology ©2012 Global Science Books

An Effective Protocol for Isolation of High-Quality RNA from Pomegranate Seeds

Abdolkarim Zarei[1*] • Zabihollah Zamani[1] • Amir Mousavi[2] • Reza Fatahi[1] • Maryam Karimi Alavijeh[1] • Bahareh Dehsara[2] • Seyed Alireza Salami[1]**

[1] Department of Horticulture Science, Faculty of Agriculture, University of Tehran, Karaj 31587, Iran
[2] National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran

Corresponding author: * zarei@ut.ac.ir, ** zzamani@ut.ac.ir

ABSTRACT

In the present study, various protocols were tested to extract RNA from different parts of pomegranate, especially the fruit. Using a modified CTAB-based procedure, high quality RNA could be extracted from different parts of pomegranate fruit. This protocol was successfully applied to isolate total RNA from the seeds of four pomegranate genotypes ('Bihaste-Ravar', 'Bihaste-Najafabad', 'Torshe-Zabol' and 'Malase-Esfahani') at five developmental stages as well as from fruit peel and leaves. Electrophoretic analysis clearly separated two ribosomal sub-units indicating no degradation of the isolated RNA. By using this protocol, the absorbance (A) ratio of 260/280 nm ranged from 1.82 to 2.06, indicating the high quality of isolated RNA with no phenolic or protein contamination. In addition, the A_{260}/A_{230} nm ratio was between 2.05 and 2.11, indicating that the extracted RNA was free of polysaccharides. The average yield of extracted total RNA was 106.42 µg/g fresh weight. Fragments of *Actin* and *18S* reference genes were successfully amplified by RT-PCR and constant expression of the *Actin* gene was confirmed by semi-quantitative RT-PCR. Using this protocol, RNA extracted from pomegranate fruit was suitable for cDNA construction and hence for subsequent molecular studies.

Keywords: aril, cDNA, electrophoretic analysis, *Punica granatum*, ribonucleic acid

INTRODUCTION

Pomegranate (*Punica granatum* L.) is an ancient fruit tree from Iran which is well adapted to drought and high temperatures, and is widely planted in arid and semi-arid regions in Asia. As the center of origin and center of diversity of this plant, Iran contains a wide range of phenotypically different pomegranate genotypes. Pomegranate has recently gained more popularity around the world due to its fruit's many medicinal properties and this has increased research regarding various aspects of this plant. New investigations revealed that this fruit has many beneficial effects on some important diseases such as cancer, cardiovascular and neurological diseases (Hora *et al*. 2003; Louis-Jeune *et al*. 2005; Seeram *et al*. 2006; Mena *et al*. 2011). Because of its medicinal as well as nutritional benefits, pomegranate has been subjected to many studies, including those involving molecular biology. Several molecular studies have been carried out in the genomics era of pomegranate (Jbir *et al*. 2008; Zarei *et al*. 2009; Zamani *et al*. 2010; Sarkhosh *et al*. 2011) but transcriptomics studies, which is an important growing field in molecular biology, is scarce on this fruit. In order to study the transcriptome, obtaining high-quality RNA is a prerequisite to succeed in subsequent steps on gene expression analysis such as quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR), differential display and microarray.

Due to the high content of metabolic compounds in woody plants, conventional procedures for RNA extraction do not result in high-quality RNA, especially for fruit tissues which contain high levels of phenolic compounds, polysaccharides, proteins and RNase (Jones *et al*. 1997). Although several protocols have been developed for extraction of total RNA from various recalcitrant plants (Meisel *et al*. 2005; Suzuki *et al*. 2008), to date no single method has been developed to isolate high-quality RNA from different parts of pomegranate. Furthermore, available commercial plant isolation kits, such as the RNeasy plant mini kit (Qiagen) and TRIzol reagent (Life Technologies Corp.) have not been designed for extraction of high-quality RNA from plant tissues that have an elevated amount of secondary metabolites (Tattersall *et al*. 2005). Pomegranate fruit contains high levels of polyphenols, polysaccharides, lipids and other secondary metabolites (El-Nemr *et al*. 1990; Rasheed *et al*. 2009). Several studies showed that these substances bind to nucleic acids during RNA isolation resulting in poor yield and also interfering with subsequent operations (Coana *et al*. 2010). This problem is more eminent when seeds are the subject of the study. Polysaccharides and other compounds frequently contaminate RNA samples from seeds and siliques (Vicient and Delseny 1999). In fact, RNA can make complexes with polysaccharides and phenolic compounds, so protocols suitable for RNA isolation from other tissues have to be modified to be applicable for seed RNA extraction. In the present study, an RNA extraction protocol was adapted to isolate high-quality RNA from different tissues of pomegranate including the aril, fruit peel, leaf, as well as the seed at different developmental stages which is easy, less expensive and does not use phenol. To our knowledge, this is the first adapted RNA isolation protocol for different parts of pomegranate.

MATERIALS AND METHODS

Plant materials

Four pomegranate genotypes, namely 'Bihaste-Ravar', 'Bihaste-Najafabad', 'Torshe-Zabol' and 'Malase-Esfahani', were selected from the National Pomegranate Collection Center of Iran in Yazd City. Fruit were harvested at different developmental stages from 20 DAF (days after flowering) to maturity (130 DAF). After the fruit opened, arils were immediately frozen in liquid nitrogen and stored at -80°C until used.

Received: 8 August, 2012. Accepted: 5 November, 2012.

Original Research Paper

Tested protocols

As a first step, we tried to select a protocol from currently existing RNA isolation methods. For this purpose, nine RNA extraction procedures were tested, among which four were commercial RNA isolation kits: Column RNA Isolation Kit (DENAzist, Tehran, Iran), RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), TRIzol Reagent (Life Technologies Corp., Carlsbad, USA), and RNX-Plus (CinnaGen, Tehran, Iran). All of the steps and reagents were applied according to the manufacturer's instructions. Also five protocols previously developed for RNA extraction from different plant organs were tested (Jones *et al*. 1997; Jaakola *et al*. 2001; Reid *et al*. 2006; Yim *et al*. 2011; Heidari-Japalaghi *et al*. 2011) (**Table 1**). All of these methods were applied as indicated by their authors. The introduced protocol described here was according to a protocol initially developed by Change *et al*. (1993) for extracting RNA from pine tree (*Pinus radiata*.) and modified by Jaakola *et al*. (2001) for RNA isolation of bilberry fruit (*Vaccinium myrtillus*) and further modified during this work for pomegranate RNA extraction as explained next.

Equipments, reagents and extraction buffers

DEPC (diethyl pyrocarbonate, Sigma-Aldrich, St. Louis, USA)-treated water was used for all solutions. All glassware, micro tubes and tips were treated with 0.1% DEPC-treated water overnight and then autoclaved twice for 45 min. All other chemicals used were of laboratory grade. Mortars and pestles were heated for at least 3 h at 200°C and chilled at -20°C before use.

Extraction buffer I: 2% CTAB (cetyltrimethylammonium bromide (Merck, Darmstadt, Germany)), 25 mM EDTA (ethylene diamine tetra acetic acid; Merck), 100 mM Tris-HCl (Merck) (pH 8.0), 2.0 M NaCl (Merck), 0.5 g/L spermidine (Sigma-Aldrich), 2% PVP (polyvinylpyrrolidone, molecular mass = 2.5 g/mol (Merck)). This solution was autoclaved (20 min at 121°C) after preparing and prior to use, 50 µg/ml proteinase K (Qiagene) was added. After extraction buffer was added to the powdered sample, 20 µl β-mercaptoethanol (Merck) was immediately added to each tube.

Extraction buffer II: 1.0 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate (SDS; Merck), this solution was autoclaved (20 min at 121°C) and before use heated to 65°C.

Other solutions (all from Merck): chloroform: isoamyl alcohol (24: 1); 10 M lithium chloride autoclaved and stored at 4°C; 70% ethanol (prepared from absolute ethanol with DEPC-treated and autoclaved water); distilled, DEPC-treated and autoclaved water; isopropanol; 3 M sodium acetate (NaOAc, pH 5.2).

Procedure

- The sample was ground in a mortar with a pestle to a fine powder (because of the hard lignified seed coat in pomegranate, it was necessary to add liquid nitrogen several times during grinding).
- Up to 100 mg of powdered tissue was transferred to a 2 ml tube and 1 ml of pre-heated (65°C) extraction buffer I was added, then immediately 20 µl β-mercaptoethanol was added to each tube. This solution was mixed thoroughly by inverting, then vortex-mixed and incubated at 65°C for 15 min. During incubation, the solution was shaken and vortex-mixed every few minutes.
- Tubes were centrifuged at 10,000 × *g* for 10 min at 4°C.
- The supernatant was transferred to a new DEPC-treated 2-ml tube, an equal volume of 24: 1 chloroform: isoamyl alcohol was added, and the suspension was vortex-mixed and centrifuged at 13,000 × *g* for 10 min (this step was repeated at least twice).
- The upper aqueous phase was transferred to a fresh tube and 1/4 volume of 10 M LiCl was added. Tubes was inverted gently to mix the solution, and incubated at 4°C overnight.
- Samples were centrifuged at 20,000 × *g* for 40 min at 4°C.
- The supernatant was decanted and the tube was gently and briefly dabbed to a paper towel.
- The pellet was washed with 500 µl of 70% ice-cold ethanol, centrifuged briefly and ethanol was decanted (this step was repeated twice).
- 300 µl of pre-warmed extraction buffer II was added to the pellet and left for 15 min to dissolve the pellet (it may be necessary to warm the tubes for a while in a water bath at 65°C).
- An equal volume of 24: 1 chloroform: isoamyl alcohol was added and, after vortexing, the mixture was centrifuged at 13,000 × *g* for 10 min at 4°C (this step was repeated twice).
- A 0.1 volume of 3 M NaOAc (pH 5.2) and 0.6 volume of isopropanol was added. This mixture was mixed gently and incubated for at least 2 h at -20°C.
- Tubes were centrifuged at 20,000 × *g* for 30 min at 4°C, the pellet was washed with ice-cold 70% ethanol and dried for 15 min on a paper towel.
- The RNA pellet was resuspended in 30 µl DEPC-treated water and DNase I (1 unit/1 µg RNA) was added.

Estimation of RNA purity, yield and integrity

The purity and concentration of the isolated RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated from the *28S* and *18S* ribosomal RNA (rRNA) bands on 1.0% formaldehyde-agarose gel after electrophoresis, staining with ethidium bromide and visualizing under UV (312 nm) light.

cDNA synthesis

Total RNA (1 µg) was reverse transcribed into cDNA with an oligo $(dT)_{18}$ primer and Reverse Transcriptase (Fermentas, Glen Burnie, USA) in 20 µl total volume as follows: 1 µl of oligo (dT), 1 µg of total RNA and up to 11.5 µl DEPC-treated water were incubated at 65°C for 10 min. Thereafter, 4 µl of M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) buffer (Fermentase), 2 µl dNTPs (1 mM final conc.), 0.5 µl (20 U) of RNase inhibitor, 1 µl (20 U) of Reverse Transcriptase, and 1 µl of DEPC-treated water were added. Reverse transcription was performed at 42°C for 60 min, followed by 10 min incubation at 70°C for inactivation and stored at -20°C until further analysis. In the case of the *18S* reference gene, 1 µl of the reverse primer (instead of oligo $(dT)_{18}$ primer) was used to synthesize the first strand of cDNA and the remaining process was the same as for the oligo $(dT)_{18}$ primer. PCR was performed by primers that were designed based on the conserved sequences for *Actin* and *18S* reference genes (**Table 2**). PCR cycles were programmed as follows: 4 min at 94°C for initial denaturing, followed by 35 cycles of 1 min denaturing at 94°C, 1 min annealing at 66°C (*Actin*) or at 58°C (*18S*), and 1 min elongation at 72°C followed by a single final cycle at 72°C for 5 min. PCR products were separated on a 1.0% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Gene expression analysis

The expression of the *Actin* reference gene was evaluated in seed transcripts of 'Bihaste-Ravar' (a soft-seed genotype) and 'Torshe-Zabol' (a hard-seed genotype), each at three developmental stages by semi-quantitative RT-PCR. PCR reactions were carried out using 2 µl of 10-fold diluted cDNA, 7.5 µl PCR kit (CinnaGene, Tehran, Iran), 1 µl of 0.5 µg of each primer, in a final volume of 15 µl. PCR was performed under the following conditions: 4 min at 94°C for initial denaturing, followed by 35 cycles of 1 min denaturing at 94°C, 1 min annealing at 66°C and 1 min elongation at 72°C followed by a single final cycle at 72°C for 5 min. The reaction was conducted in triplicate and 5 µl of PCR products were separated on 1.0% agarose gel stained with ethidium bromide and observed under UV light.

RESULTS AND DISCUSSION

Nine different RNA extraction methods, including CTAB-based methods, as well as commercial plant RNA extraction kits, were used to isolate RNA from pomegranate seed and aril. According to our results, most of these methods could not isolate RNA of suitable quality and quantity for cDNA synthesis and subsequent RT-PCR application. Commercial RNA isolation kits did not result in RNA extract from

Table 1 All of the tested protocols in this study along with a summary of their procedures as well as their plant species.

Tested protocol	Procedure	Plant species and target tissue
Column RNA isolation kit (DENAzist, Iran)	Add 1ml DR1 buffer to 50 mg grounded tissue, homogenize and centrifuge at 10,000 rpm for 10 min. Add 200 µl chloroform to the supernatant, vortex for 15 seconds, incubate at room temperature for 3 min, centrifuge at 12,000 g for 15 min. Transfer the top phase into a new tube and add equal to half of the volume of the aqueous phase from 100% ethanol. Centrifuge at 10,000 rpm in a spin column for 1 min, discard the flow-through. Add 500 µl DR2 to the spin column, centrifuge at 10,000 rpm for 1 min and discard the flow-through. Add 700 µl DR3 to the spin column, centrifuge at 10,000 rpm for 1 min, and discard the flow-through. Add 50-100 µl DR4 to the center of the column, incubate at room temperature for 2 min and centrifuge at 10,000 rpm for 2 min.	-
RNeasy Plant Mini Kit (Qiagen, Germany)	Add 450 µl buffer RLT to 100 mg grounded tissue, vortex and transfer to a QIAshredder spin column, then centrifuge for 2 min at full speed. Transfer the supernatant to a new tube, add 0.5 volume of ethanol (96–100%), and mix immediately. Transfer the sample to an RNeasy spin column placed in a 2 ml collection tube and centrifuge for 15 s at 10,000 rpm and discard the flow-through. Add 700 µl buffer RW1 to the RNeasy spin column and centrifuge for 15 s at 10,000 rpm and discard the flow-through. Add 500 µl buffer RPE, centrifuge for 15 s at 10,000 rpm and discard the flow-through (repeat this step). Place the RNeasy spin column in a new 1.5 ml collection tube, add 30-50 µl RNase-free water to the spin column membrane, centrifuge for 1 min at 10,000 rpm.	-
TRIzol Reagent (Life Technologies USA)	Add 1 ml of TRIzol reagent to the tube containing 50 mg grounded tissue, vortex for 1 min and incubate at RT for 5 min Add 200 µL of chloroform, shake vigorously, incubate at RT for 2 min and centrifuge at 12,000 g for 15 min. Add 200 µL of chloroform to the supernatant, shake vigorously, incubate at RT for 2 min and centrifuge at 12,000 g for 15 min. Add 600 µL isopropanol to the supernatant, mix and incubate for 10 min at RT, centrifuge at 12,000 g for 10 min. Wash the pellet with 70% ethanol, centrifuge at 7,500 g for 5 min, discard supernatant, air dry for 15 min and add 100 µL H_2O.	-
RNX™-PLUS (CinnaGen, Iran)	Add 1 ml of RNX™-PLUS solution to 100 mg grounded tissue, vortex and incubate at RT for 5 min. Add 200 µL chloroform, mix and centrifuge at 12,000 rpm for 15 min. Transfer the supernatant and add an equal volume of isopropanol, gently mix and incubate on ice for 15 min. Centrifuge at 12,000 rpm for 15 min, wash the pellet with 70% ethanol, let the pellet dry and add 50 µL DEPC-H_2O.	-
Jones *et al*. 1997	Add 20 ml buffer:phenol (1:1) preheated to 80°C to 4 g grounded tissue, vortex and centrifuge at 20,000 g for 10 min. Remove supernatant, add equal volume of chloroform:isoamyl alcohol, vortex and centrifuge at 20,000 g for 10 min. Remove supernatant, add 1/3 volume of 12 M LiCl, mix and incubate at 4°C overnight. Centrifuge at 20,000 g for 90 min, decant the supernatant, then wash the pellet with 70% ethanol and dry it. Resuspend the pellet in 1 ml ddH_2O, centrifuge at 8,000 g for 5 min and transfer the supernatant to a new tube. Add 1 ml of β-mercaptoethanol, then add LiCl to a concentration of 0.8 M and incubate at -20°C for 2 h. Centrifuge at 20,000 g for 30 min, wash the pellet with 70% ethanol, resuspend the dried pellet in 100 µL DEPCH_2O.	*Rubus idaeus* fruit
Reid *et al*. 2006	Ground 1 g of tissue and add 20 ml extraction buffer, shake and incubate at 65°C for 10 min. Extract twice with equal volume of chloroform:isoamyl alcohol (24:1) then centrifuge at 3,500 g for 15 min. Centrifuge aqueous phase at 30,000 g for 20 min, add 0.1 volume 3 M NaOAc (pH 5.2) and 0.6 volume isopropanol to the supernatant, mix and store at -80°C for 30 min. Centrifuge at 3,500 g for 30 min, dissolve the pellet in 1 ml TE (pH 7.5). Add 0.3 volume of 8 M LiCl and store at 4°C overnight. Centrifuge at 20,000 g for 30 min, then wash with 70% ethanol, air dry and dissolve in 100 µL DEPC-H_2O.	*Vitis vinifera* fruit
Heidari-Japalaghi *et al*. 2011	Add 1 ml of extraction buffer to 100 mg grounded tissue, incubate at 65°C for 20-30 min. Add an equal volume of chloroform:isoamyl alcohol (24:1), shake and centrifuge at 14,000 rpm for 5 min. Transfer the supernatant to a new tube and extract with chloroform:isoamyl alcohol (24:1). Collect the supernatant, add 0.1 volume of 3 M NaOAc pH 5.2 and an equal volume of isopropanol then store at -80°C for 30 min. Centrifuge at 14,000 rpm for 30 min, dissolve the pellet in 200 µl water, add 0.3 volume of 8M LiCl and store at 4°C overnight. After centrifugation at 14,000 rpm for 30 min, wash the pellet with 70% ethanol, air dry and dissolve in 100 µl water.	*Malus domestica* *Prunus persica* *Prunus avium* *Vitis vinifera* *Prunus armeniaca* fruit
Yim *et al*. 2011	Add 5 ml extraction buffer to 100 mg grounded tissue, vortex and incubate at 65°C for 20 min. Cool sample, add 0.6 volume chloroform, vortex and incubate for 5 min, then centrifuge at 8,000 rpm for 20 min. Pipette off the supernatant to a new tube and repeat previous step. Add 0.3 volume of 8 M LiCl, mix gently and incubate at -80°C overnight. Centrifuge at 8,000 rpm for 20 min, wash the pellet with 75% ethanol, air dry for 10 min and dissolve in 100 µl water.	*Arachis hypogaea* fruit
Jaakola *et al*. 2001	Weight 10 × 100 mg of the powdered tissue, transfer to 1.5 ml tubes and add 750 µl of the extraction buffer to each tube. Incubate at 65°C for 10 min, centrifuge at 10,000 g for 10 min. Extract the supernatant twice with an equal volume of chloroform:isoamyl alcohol, add ¼ volume 10 M LiCl store overnight at 4°C. Centrifuge at 18,000 g for 20 min, discard the supernatant, let the pellet dry and wash it with 70% ethanol and centrifuge. Dissolve the pellet in 100 µl SSTE, then combine 10 samples. Extract the contents with an equal volume of phenol:choloroform:isoamyl alcohol (25:24:1). Extract the contents with an equal volume of chloroform:isoamyl alcohol (24:1). Add two volumes of absolute ethanol to the supernatant and store at -20°C for 2 h. Centrifuge at 18,000 g for 20 min, wash the pellet with 70% ethanol, dry it and add DEPC-H_2O.	*Vaccinium myrtillus* fruit

Table 2 Tested reference genes on RNA extracts of pomegranate, sequence of primers, and the size of amplicon fragments.

Reference gene	Primer sequence	Fragment size
Actin	Forward: 5'-GGAGAAGATTTGGCATCA-3'	660 bp
	Reverse: 5'-CACTTTCTACAATGAG-3'	
18S	Forward: 5'-TTCGGGATCGGAGTAATGATTAA-3'	620 bp
	Reverse: 5'-GCCCAGAACATCTAAGGGCATCACAGA-3'	

Table 3 Means comparison of quality (as absorbance ratios) and yield of isolated RNA from different pomegranate tissues at different developmental stages.

Pomegranate tissue	Absorbance at 260/280 nm**	Absorbance at 260/230 nm*	Concentration ng/µl**	RNA yield µg/g FW**
Seed at 20 DAF	2.06 ± 0.09 a	2.11 ± 0.10 abc	418.85 ± 99.87 ab	113.09 ± 29.95 ab
Seed at 40 DAF	2.03 ± 0.06 abc	2.20 ± 0.24 a	469.53 ± 180.56 a	126.77 ± 48.75 a
Seed at 60 DAF	2.04 ± 0.07 abc	2.23 ± 0.18 a	414.49 ± 170.49 ab	111.92 ± 46.04 ab
Seed at 80 DAF	1.92 ± 0.1 cb	2.03 ± 0.20 ab	308.21 ± 112.31 ab	83.24 ± 30.32 ab
Seed at 130 DAF	1.97 ± 0.05 ab	2.10 ± 0.15 abc	359.48 ± 122.17 ab	97.06 ± 32.99 ab
Aril at maturity stage	2.03 ± 0.12 abc	2.18 ± 0.13 abc	407.32 ± 131.57 ab	109.98 ± 35.53 ab
Fruit peel	1.94 ± 0.21 c	2.08 ± 0.9 bc	287.62 ± 150.24 b	77.66 ± 40.56 b
Leaf	1.82 ± 0.09 d	2.05 ± 0.03 c	97.81 ± 27.49 c	26.41 ± 7.42 c

The values are average of at least 12 replications (four pomegranate genotypes each of them three replications). (Means sharing the same letters indicate no significant differences using DMRT at 5% level.**: significant at 1% level; * significant at 5% level.)

Table 4 RNA yield of different pomegranate genotypes and tissues at different developmental stages.

Pomegranate genotypes/tissue	RNA yield at 20 DAF µg/g FW	RNA yield at 40 DAF µg/g FW	RNA yield at 60 DAF µg/g FW	RNA yield at 80 DAF µg/g FW	RNA yield at 130 DAF µg/g FW
Bihaste-Ravar	104.29 ± 55.29	127.09 ± 65.01	129.56 ± 64.45	88.96 ± 35.09	129.14 ± 40.12
Bihaste-Najafabad	108.69 ± 23.7	122.89 ± 57.56	108.63 ± 37.56	85.131 ± 24.29	94.8 ± 31.16
Torshe-Zabol	118.15 ± 7.73	121.28 ± 54.01	109.29 ± 12.53	85.625 ± 9.73	80.73 ± 31.16
Malase-Esfahani	120.94 ± 3.43	136.26 ± 43.74	100.152 ± 30.62	72.99 ± 19.14	83.5 ± 17.13
Aril at maturity stage	-	-	-	-	109.98 ± 35.53
Fruit peel	-	-	-	-	77.66 ± 40.56
Leaf	-	-	-	-	26.41 ± 7.42

pomegranate seeds. This might be because these kits have not been designed for isolation of high-quality RNA from plant tissues rich in secondary metabolites (Tattersall *et al*. 2005). Among the other methods, three of them did not extract any RNA or possibly degraded it after extraction from tissue. Among all these protocols, a method described by Yim *et* al. (2011) for nucleic acid isolation (both DNA and RNA) from peanut (*Arachis hypogaea*) seed, extracted RNA of very low quality and quantity along with a copious amount of contaminating genomic DNA, which was not suitable for cDNA synthesis. Finally, a protocol that had been described by Chang *et al*. (1993) for RNA isolation from pine tree and modified by Jaakola *et al*. (2001) for RNA isolation from bilberry fruit, was the only procedure that could isolate workable RNA from pomegranate seeds and was thus better than the other methods. However, this protocol is not only very laborious but also needs a high amount of starting plant material (almost ten times more than the optimized procedure), more equipment as well as chemicals. Furthermore, it uses some hazardous substances like phenol. In fact, in this protocol, 10 samples of each starting plant material must be extracted and in the final step these 10 tubes must be pooled to have a measurable amount of RNA. Therefore, after some modifications, the modified version of this procedure was optimized for high-quality RNA isolation from pomegranate fruit. One of the advantages of this protocol might be the use of two extraction buffers, one containing CTAB and the other containing SDS. This combination makes the protocol more effective for getting rid of proteins, especially RNases. Moreover, because of the second round of extraction with SDS, the extracted RNA exhibits considerable high quality.

The absence of phenol in the extraction procedure is also one of the advantages of this modified method, which not only avoids this hazardous compound, but also reduces the potential damage to poly A (+) RNA (Chang *et al*. 1993). Proteinase K was used to digest protein contamination as well as to protect the nucleic acids from the activity of nucleases. The amount of extraction buffer II was increased three-fold in each tube, making it a better solvent for the pellet and subsequently generating more suspension volume. Also, in the final step, instead of ethanol, isopropanol and 3 M sodium acetate were used to precipitate RNA, which caused the final concentration of the isolated RNA to be relatively high. To eliminate genomic DNA contamination, total RNA was treated with DNase I according to the manufacturer's instruction. Excess DNase I was later deactivated and eliminated by precipitation of the ribonucleic acid in absolute ethanol and 3M sodium acetate at pH 5.2.

CTAB-based methods have been previously used successfully for RNA extraction from different organs of several woody plants such as apple (*Malus domestica*) (Gasic *et al*. 2004), peach (*Prunus persica*) (Meisel *et al*. 2005), and grapevine (*Vitis vinifera*) (Gambino *et al*. 2008). In the present work, after testing various methods as well as commercial plant RNA extraction kits, we concluded that, compared to guanidinium thiocyanate, which is the main component of many commercial kits and which allows RNA isolation from friable and young tissues such as *in vitro* plants (Gambino *et al*. 2008), CTAB and SDS can extract RNA more efficiently from woody plants which have high levels of (poly)phenols, polysaccharides and other compounds that interfere with RNA extraction.

Quantity and quality of extracted RNA

Using the described protocol, the RNA obtained was of high quality and could be obtained in good quantity (**Table 3**). Gel electrophoresis of total RNA showed two distinct 28S and 18S ribosomal bands without smearing, representing intact isolated RNA (**Fig. 1**). The average yield of total RNA was 106.42 µg/g fresh weight. This amount is comparable with RNA yield extracted from other plants such as sorghum (*Sorghum bicolor*) seeds (100 µg/g) (Sharma *et al*. 2003), *Arabidopsis* seed (107 µg/g) (Meng and Feldman 2010), peanut seeds (61.15 µg/g) (Yin *et al*. 2011), *Lycium barbarum* (fructus lycii) fruit (86.5 µg/g) (Tao *et al*. 2011) and blueberry (*Vaccinum corymbosum*) fruit (104.14 and 130.01 µg/g) (Vashisth *et al*. 2011).

The highest quantity of total RNA was extracted from seeds at 40 DAF and afterward at 20 DAF (**Table 4**). At these stages, the woody portion of seeds has not yet formed,

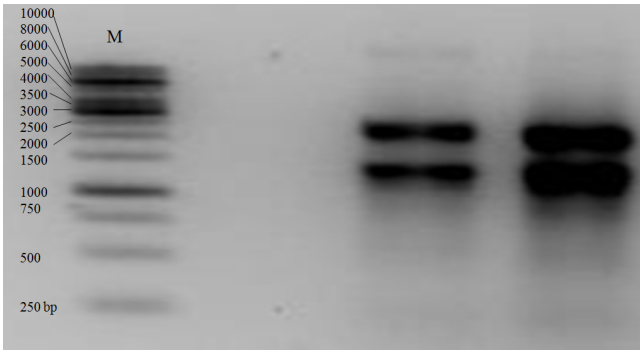

Fig. 1 Electrophoretic analysis of RNA isolated from seeds of pomegranate showing intact 28S and 18S ribosomal bands. M: 1Kb molecular weight marker.

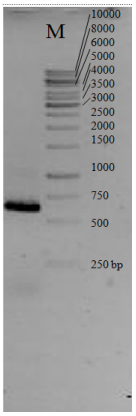

Fig. 2 Reverse transcript PCR amplification of mRNA of *Actin* reference gene using total RNA isolated by modified protocol. M: 1 kb molecular weight marker.

supporting the importance of fully pulverizing tissue for a better outcome. Also, at the preliminary stages of seed development, reserve materials like polysaccharides and lipids have not yet formed in the seed. Pomegranate seed is a rich source of various fatty acids (Sassano *et al*. 2009) which may interfere with RNA extraction (Sangha *et al.* 2010) at the maturity stage (130 DAF). The optimized protocol was successfully tested on pomegranate aril, fruit peel and leaf, although results indicated that among the tested tissues, leaf produced the least amount (97.81 ng/µl) of total RNA. As previously reported (Jones *et al*. 1997; Sivakumar *et al*. 2007), RNA extraction protocols should be optimized for each tissue. Unlike seed, aril and peel, leaf was sampled at fall, at the end of the growing season when leaves were old. At this time pomegranate trees are entering the dormancy phase in Iran, and the physiological state as well as gene expression is low.

The absorbance ratio at 260/280 ranged from 1.82 to 2.06 indicating the high quality of RNA and no protein contamination. Also the A_{260}/A_{230} ratio was between 2.05 and 2.11, which indicates that there were no polysaccharides in the extracted RNA.

Reference gene amplification

After cDNA synthesis from mRNA using oligo $(dT)_{18}$ primer, the PCR reaction was performed using the *Actin* reference gene. The primers of this gene were designed in a manner such that an intron existed in the amplicon, so if an amplified fragment had been derived from the genomic DNA contamination, its size would have been larger (700 versus 660 bp). Thus, after separation in the gel, the amplified fragment from genomic DNA should be located higher than for cDNA (**Fig. 2**). RT-PCR from *18S* rRNA produced a fragment at the predicted size (620 bp) (**Fig. 3**). A negative control was used that included all the components for the RT reaction, but excluded reverse transcriptase.

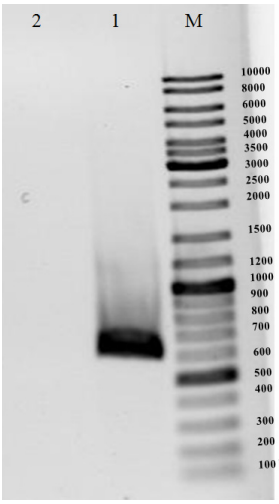

Fig. 3 Reverse transcript PCR amplification of *18S* rRNA reference gene. Lane 1: *18S* fragment, Lane 2: minus control (without RT enzyme), (M: DNA ladder mix).

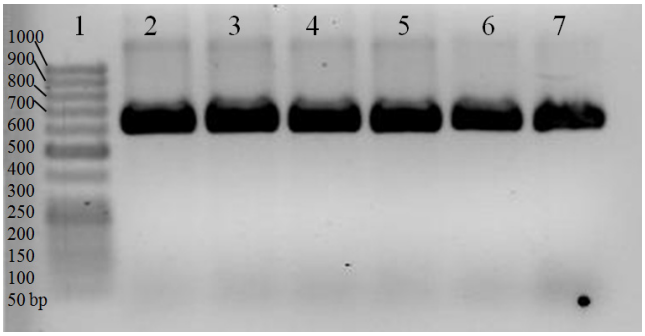

Fig. 4 Semi-quantitative PCR of *Actin* reference gene from cDNA of seeds from two pomegranate genotypes at three developmental stages. 1: 50 bp molecular weight marker, 2, 4, and 6: 'Bihaste-Ravar' at 20, 80, and 130 DAF, respectively; 3, 5, and 7: 'Torshe-Zabol' at 20, 80, and 130 DAF, respectively.

These results indicate that the DNA fragments obtained are produced from the transcripts for each gene, which represents the purity and intactness of isolated RNA using this procedure, which makes it suitable to be applied for downstream reactions.

Semi-quantitative PCR

In addition to gel electrophoresis analysis of ribosomal bands, the intactness of isolated RNA can be assessed by expression analysis of genes in various samples (Heidari-Japalaghi *et al*. 2011). The expression of the *Actin* reference gene was analyzed by semi-quantitative RT-PCR. Ideally, the conditions of the experiment should not influence the expression of the reference gene (Schmittgen and Zakrajsek 2000). Results of *Actin* reference gene amplification from seed transcripts of two pomegranate genotypes at three developmental stages displayed no variation in semi-quantitative PCR (**Fig. 4**). Since the *Actin* reference gene was expressed in all of the tested samples with minimal variations, it is a good reference for gene expression analysis in pomegranate.

ACKNOWLEDGEMENTS

The authors thank Dr. Jaime A. Teixeira da Silva for improving the language of the manuscript. The authors also thank the University of Tehran as well as the National Institute of Genetic Engineering and Biotechnology (NIGEB) of Iran for supporting this investigation.

REFERENCES

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11**, 113-116

Coana YP, Parody N, Fernandez-Caldas N, Alonso C (2010) A modified protocol for RNA isolation from high polysaccharide containing *Cupressus arizonica* pollen. Applications for RT-PCR and phage display library construction. *Molecular Biotechnology* **44**, 127-132

El-Nemr SE, Ismail IA, Ragab M (1990) Chemical composition of juice and seeds of pomegranate fruit. *Food/Nahrung* **34**, 601-606

Gambino S, Perrone I, Gribauto I (2008) A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochemical Analysis* **19**, 520-525

Gasic K, Hernandez A, Korban SS (2004) RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. *Plant Molecular Biology Reporter* **22**, 437-438

Heidari-Japalaghi R, Haddad R, Ghasem-Ali G (2011) Rapid and efficient isolation of high quality nucleic acids from plant tissues rich in polyphenols and polysaccharides. *Molecular Biotechnology* **49**, 129-137

Hora JJ, Maydew ER, Lansky EP, Dwivedi C (2003) Chemopreventive effects of pomegranate seed oil on skin tumor development in CD1 mice. *Journal of Medicinal Food* **6**, 157-161

Jaakola L, Pirttila AM, Halonen M, Hohtola A (2001) Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. *Molecular Biotechnology* **19**, 201-203

Jbir R, Hasnaoui N, Mars M, Marrakchi M, Trifi M (2008) Characterization of Tunisian pomegranate (*Punica granatum* L.) cultivars using amplified fragment length polymorphism analysis. *Scientia Horticulturae* **115**, 231-237

Jones CS, Lannetta PPM, Woodhead M, Davies HV, McNicol RJ, Taylor MA (1997) The isolation of RNA from raspberry (*Rubus idaeus*) fruit. *Molecular Biotechnology* **8**, 219-221

Louis-Jeune MA, Kumi-Diaka J, Brown J (2005) Anti cancer activities of pomegranate extracts and genistein in human breast cancer cells. *Journal of Medicinal Food* **8**, 469-475

Meisel L, Fonseca B, Gonzalez S, Baeza-Yates R, Cambiazo V, Campos R, Gonzalez M, Orellana A, Retamales J, Silva H (2005) A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. *Journal of Biological Research* **38**, 83-88

Mena P, Girones-Vilaplana A, Moreno DA, Garcia-Viquera C (2011) Pomegranate fruit for health promotion: Myths and realities. *Functional Plant Science and Biotechnology* **5**, 33-42

Meng L, Feldman L (2010) A rapid TRIzol-based two-step method for DNA-free RNA extraction from *Arabidopsis* siliques and dry seeds. *Biotechnology Journal* **5**, 183-186

Rasheed Z, Akhtar N, Anbazhagan AN, Ramamurthy R, Shukla M, Haqqi TM (2009) Polyphenol-rich pomegranate fruit extract (POMx) suppresses PMACI-induced expression of pro-inflammatory cytokines by inhibiting the activation of MAP kinases and NF-κB in human KU812 cells. *Journal of Inflammation* **6**, 1-12

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biology* **6**, 27

Sangha JS, Gu K, Kaur J, Yin Z (2010) An improved method for RNA isolation and cDNA library construction from immature seeds of *Jatropha curcas* L. *BMC Research Notes* **3**, 126

Sarkhosh A, Zamani Z, Fatahi R, Hassani ME, Wiedow C, Buck E, Gardiner SE (2011) Genetic diversity of Iranian soft-seed pomegranate genotypes as revealed by fluorescent-AFLP markers. *Physiology and Molecular Biology of Plants* **17**, 305-311

Sassano G, Sanderson P, Fran X, Groot P, Van-Straalen P, Bassaganya-Riera J (2009) Analysis of pomegranate seed oil for the presence of jacaric acid. *Journal of the Science of Food and Agriculture* **89**, 1046-1052

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: Validation by real time, quantitative RT-PCR. *Journal of Biochemical and Biophysical Methods* **46**, 69-81

Seeram NP, Schulman RN, Heber D (2006) *Pomegranates, Ancients Roots to Modern Medicine*, CRC Press, New York, 244 pp

Sharma AD, Gill PK, Singh P (2003) RNA isolation from plant tissues rich in polysaccharides. *Analytical Biochemistry* **314**, 319-321

Sivakumar S, Franco OL, Thayumanavan B (2007) Isolation of RNA from polysaccharide-rich seeds. *Preparative Biochemistry and Biotechnology* **37**, 323-332

Suzuki Y, Mae T, Makino A (2008) RNA extraction from various recalcitrant plant tissues with a cethyltrimethylammonium bromide-containing buffer followed by an acid guanidium thiocyanate-phenol-chloroform treatment. *Bioscience, Biotechnology, and Biochemistry* **72**, 1951-1953

Tao N, Gao Y, Liu Y, Zhang J (2010) Extraction of high-quality RNA and construction of a cDNA library from fruits of *Lycium barbarum* Linnaeus (fructus lycii). *Biotechnology and Biotechnological Equipment* **24**, 1569-1572

Tattersall EAR, Ergul A, AlKayal F, DeLuc L, Cushman JC, Cramer GR (2005) Comparison of methods for isolating high-quality RNA from leaves of grapevine. *American Journal of Enology and Viticulture* **56**, 400-406

Vashisth T, Johnson LK, Malladi A (2011) An efficient RNA isolation procedure and identification of reference genes for normalization of gene expression in blueberry. *Plant Cell Reports* **30**, 2167-2176

Vicient CM, Delseny M (1999) Isolation of total RNA from *Arabidopsis thaliana* seeds. *Analytical Biochemistry* **268**, 412-413

Yin D, Liu H, Zhang X, Cui D (2011) A protocol for extraction of high-quality RNA and DNA from peanut plant tissues. *Molecular Biotechnology* **49**, 187-191

Zamani Z, Zarei A, Fatahi R (2010) Characterization of progenies derived from pollination of pomegranate cv. Malase-Tourshe-Saveh using fruit traits and RAPD molecular marker. *Scientia Horticulturae* **124**, 67-73

Zarei A, Zamani Z, Fatahi R (2009) Evaluation of genetic relationships among some Persian cultivated and a wild pomegranate accession using RAPDs and SSRs molecular markers. *Horticulture, Environment, and Biotechnology* **50**, 224-232